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Therapeutic Potential of Robusta Green Coffee Extract for Inflammatory Bowel Disease: An In Vitro Mouse Model

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Abstrak:
Pendahuluan: Penyakit radang usus (*Inflammatory Bowel Disease*/IBD) merupakan peradangan kronis pada saluran pencernaan yang ditandai oleh respons imun abnormal terhadap mikrobiota usus. Penelitian ini bertujuan untuk mengevaluasi potensi anti-inflamasi ekstrak kopi hijau Robusta/ RGC (*Coffea canephora* var. Robusta) menggunakan model peradangan in vitro yang melibatkan makrofag dan sel epitel usus. **Metode**: Sel primer makrofag dan epitel usus diisolasi dari rongga peritoneal dan usus mencit jantan (*Mus musculus*), diinduksi menggunakan lipopolisakarida (1 µg/mL) pada sumuran, kemudian diberi perlakuan ekstrak dengan varian konsentrasi (normal, LPS, 30 µg/mL, 50 µg/mL, dan 70 µg/mL). Setiap perlakuan dilakukan tiga kali ulangan (n=3). Viabilitas sel dianalisis menggunakan metode MTT *assay*, sedangkan ekspresi sitokin proinflamasi TNF-α diamati menggunakan *flow cytometry*. Analisis statistik dilakukan menggunakan ANOVA *One Way* yang diuji lanjut pada *post hoc Tukey* dan *Duncan's*. **Hasil**: Penelitian menunjukkan bahwa ekstrak kopi hijau Robusta secara signifikan (p=0,00) meningkatkan viabilitas makrofag dan sel epitel usus serta menurunkan ekspresi TNF-α pada makrofag distimulasi LPS. Konsentrasi 30 µg/mL menunjukkan viabilitas dan ekspresi TNF-α makrofag yang optimal, sedangkan konsentrasi 50 µg/mL menunjukkan pengaruh yang optimal pada viabilitas sel epitel usus. **Kesimpulan**: Berdasarkan hasil penelitian, dapat disimpulkan bahwa ekstrak kopi hijau Robusta mungkin memiliki potensi sebagai agen antiinflamasi dan imunomodulator untuk penanganan IBD, meskipun masih diperlukan validasi lebih lanjut melalui melalui studi in vivo.
Kata kunci: Anti-inflamasi; Epitel usus; In vitro; Kopi hijau Robusta; Makrofag.

Abstract:
Introduction: Inflammatory Bowel Disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract characterized by an abnormal immune response to gut microbiota. This study aimed to evaluate the anti-inflammatory potential of Robusta green coffee/ RGC (Coffea canephora var. Robusta) extract using an in vitro inflammation model involving macrophages and intestinal epithelial cells. **Methods**: Primary macrophages and intestinal epithelial cells were isolated from the peritoneal cavity and intestines of male Mus musculus, induced with lipopolysaccharide (LPS) (1 µg/mL) in the well plate and treated with Robusta green coffee extract with variance concentrations (normal, LPS, 30 µg/mL, 50 µg/mL, and 70 µg/mL). Each treatment was conducted in triplicate (n=3). Cell viability was assessed using the MTT assay, while the expression of the pro-inflammatory cytokine TNF-α was analyzed by flow cytometry. Statistical analysis was performed using One-way ANOVA followed by Tukey's post hoc test. **Results**: Robusta green coffee extract significantly (p=0.00) improved the viability of both macrophages and intestinal epithelial cells. Moreover, it reduced TNF-α expression in LPS-stimulated macrophages. A concentration of 30 µg/mL showed optimal effects on macrophage viability and TNF-α expression, while 50 µg/mL showed optimal effects on intestinal epithelial cell viability. **Conclusions**: Robusta green coffee extract may have potential anti-inflammatory and immunomodulatory properties for managing Inflammatory Bowel Disease (IBD), pending further validation through in vivo studies.
Keywords: *Anti-inflammatory; Intestinal epithelium; In vitro; Macrophages; Robusta green coffee.*

1. Introduction

Inflammatory Bowel Disease (IBD) is a chronic, relapsing inflammatory disorder of the gastrointestinal tract that significantly compromises patients' quality of life and represents an increasing global health burden. The two primary forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC), are characterized by sustained intestinal inflammation resulting from an abnormal immune response to intestinal microbiota (1,2). One of the key pathogenic mechanisms in IBD involves the excessive production of pro-inflammatory cytokines, particularly Tumor Necrosis Factor-α (TNF-α), stimulated by Gram-negative bacteria such as *Escherichia coli* (3,4). Elevated TNF-α levels have been shown to significantly trigger apoptosis in both macrophages and intestinal epithelial cells, thereby exacerbating tissue damage and intestinal inflammation in IBD cases (5,6). This inflammatory cascade is further amplified by the activation of Toll-like Receptor 4 (TLR-4) by lipopolysaccharide (LPS), which leads to MyD88-dependent signaling, nuclear factor kappa B (NF-κB) activation, and the production of reactive oxygen species (ROS), collectively sustaining the chronic inflammatory response (7,8).

In recent years, extensive research has explored the potential of natural bioactive compounds as adjunct or alternative therapies to modulate chronic intestinal inflammation in IBD (9,10). Among these, Robusta Green Coffee (RGC) bean extract (*Coffea canephora*) has attracted attention due to its pharmacological properties, particularly its antioxidant and anti-inflammatory effects. It contains various bioactive compounds such as phenolic acids, polyphenols, caffeine, and chlorogenic acid, which have been reported to suppress oxidative stress and pro-inflammatory cytokine production (11-13). Previous in vivo studies demonstrated that RGC extracts effectively reduced TNF-α production in LPS-induced macrophage models and protected against histopathological damage in intestinal epithelial tissue (14,15).

Although previous studies have explored the anti-inflammatory effects of RGC extract in single-cell models and through histological examination of intestinal tissue (in vivo), comprehensive investigations using in vitro models involving multiple relevant cell types remain limited. A model utilizing both peritoneal macrophages and intestinal epithelial cells offers a more physiologically relevant simulation of the intestinal environment in IBD, allowing for a deeper understanding of intercellular interactions in the inflammatory response. Therefore, this study aimed to evaluate the anti-inflammatory and immunomodulatory effects of RGC extract on TNF-α production and cell viability in LPS-induced peritoneal macrophages and intestinal epithelial cells isolated from Mus musculus. The findings from this research are expected to provide novel insights into the therapeutic potential of plant-derived natural compounds as complementary strategies in the management of IBD.

2. Materials and Methods

2.1 Study Design

This study was an experimental laboratory using an in vitro model. Macrophages and intestinal epithelial cells were isolated from male mice and cultured under controlled laboratory conditions. The cells were divided into six groups: a normal control (without LPS induction and treatment), a negative control (induced by lipopolysaccharide (LPS) without treatment), and four treatment groups induced by LPS followed by exposure to RGC extract at concentrations of 30, 50, and 70 µg/mL. Cell viability was assessed using the MTT assay, while TNF-α expression was analyzed by flow cytometry. Each treatment was performed in triplicate, and data were collected for statistical analysis.

2.2. Ethical Statement

The study protocol was approved by the Ethics Committee for Health Research, Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Malang, Indonesia. a (approval number: 016/EC/KEPK-FKIK/2018).

2.3. Materials

The primary material used in this study was RGC beans (*Coffea canephora* var. Robusta). The experimental animals consisted of two male Balb/c mice (Mus musculus), aged 6–8 weeks, certified pathogen-free. The reagents

and materials employed in this study included RPMI-1640 medium, Phosphate Buffered Saline (PBS) without calcium and magnesium, physiological saline solution (0.98% NaCl), complete medium supplemented with 10% Fetal Bovine Serum (FBS), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS) from Escherichia coli, sodium bicarbonate (TC230), penicillin, streptomycin, distilled water (DI water), MTT assay kit, CD11b antigen, TNF-α-PE antigen, intracellular staining buffer (PWB), trypan blue, Giemsa stain, and aquadest.

The equipment utilized in this study included a Laminar Air Flow (LAF) cabinet, 5% CO_2 incubator, inverted microscope, cell counter, flow cytometry FACS Calibur (Thermo Fischer, Massachusetts, United States), ELISA reader, autoclave, laboratory oven, analytical balance, 96-well flat-bottom culture plates (F-base), and refrigerated centrifuge.

2.4. Procedures

2.4.1. Extract preparation

A total of 100 grams of RGC (*Coffea canephora* var. Robusta) powder was extracted using a maceration method with distilled water as the solvent. The extraction was performed at a ratio of 1:6 by immersing the coffee powder in 600 mL of distilled water preheated to 70°C for 2 hours using a hot plate stirrer. The resulting extract was then concentrated and dried using a freeze-drying technique to obtain a powder form for use in subsequent experiments. This method was adapted from a previous study.

2.4.2. Isolation of Macrophages and Intestinal Epithelial Cells

Peritoneal macrophages were isolated from euthanized mice by cervical dislocation. After sterilizing the abdominal surface with 70% ethanol, 10 mL of cold RPMI medium was injected into the peritoneal cavity using an 18G syringe. The peritoneal fluid was collected, and centrifuged, and the resulting cell pellet was used for macrophage isolation. Intestinal epithelial cells were isolated from the small intestines of mice. The intestines were cleaned with cold PBS, chopped into small pieces, and incubated in RPMI medium supplemented with penicillin-streptomycin at 37°C. The intestinal crypts were then isolated, suspended in RPMI medium containing 10% FBS, and cultured at 37°C in a humidified atmosphere with 5% CO_2 until reaching 50–70% confluence.

2.4.3. Treatment Procedure

The cultured macrophages and intestinal epithelial cells were induced with 1 µg/mL lipopolysaccharide (LPS) for 1 hour to trigger an inflammatory response. Subsequently, the cells were treated with green coffee extract at concentrations of 30 µg/mL, 50 µg/mL, and 70 µg/ml. Each treatment was conducted in four replicates.

2.4.4. Cell Viability Assay

The viability of peritoneal macrophages and intestinal epithelial cells was assessed using the colorimetric MTT assay. Following incubation, the MTT reagent was added to the culture wells and incubated for 3–4 hours to allow formazan crystal formation. The resulting formazan crystals were dissolved with 10% SDS in 0.01 N HCl, and the absorbance was measured at a wavelength of 490–550 nm using an ELISA reader.

2.4.5. Measurement of Cytokine Numbers by Flow Cytometry

TNF-α production by peritoneal macrophages was analyzed using flow cytometry. Harvested macrophage cells were washed with PBS and stained extracellularly with a CD11b antibody to identify macrophage populations. After extracellular staining, the cells were fixed, permeabilized, and stained intracellularly with a TNF-α antibody. Flow cytometric analysis was then performed to quantify TNF-α expression in CD11b-positive macrophages.

2.5. Statistical Analysis

Data of cell viability from MTT assay of peritoneal macrophages and intestinal epithelial cells, as well as the relative expression of the proinflammatory cytokine TNF-α from CD11b+ peritoneal macrophages, were expressed as mean ± standard error of the mean (SEM). All data were initially tested for normality using the Kolmogorov-Smirnov test and for homogeneity of variance using Levene's test. If the data satisfied both assumptions, a one-way analysis of variance (ANOVA) was conducted, followed by Tukey's and Duncan's multiple range post-hoc tests to determine significant differences between treatment groups at a significance level of $p < 0.05$. The expression levels

of TNF-α from flow cytometry analysis were processed using CellQuest software (BD Biosciences, San Jose, CA, USA). All statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Effect of Robusta Green Coffee Extract on Cell Viability

To evaluate the immunomodulatory potential of Robusta Green Coffee (RGC) extract, cell viability assays were performed on LPS-induced peritoneal macrophages and intestinal epithelial cells. Cell viability served as a key indicator of cellular health and functional restoration under inflammatory stress.

As shown in **Figures 1a and 1b**, LPS exposure significantly reduced the viability of both cell types compared to normal controls, confirming effective induction of inflammation. Treatment with RGC extract at various concentrations (30, 50, and 70 µg/mL) led to a notable improvement in cell viability. The most pronounced protective effect was observed at 30 µg/mL, which restored macrophage viability to 80.19% ± 11.07 and epithelial cell viability to 87.84% ± 6.80. These findings suggest that RGC extract enhances the survival of both immune and barrier-forming intestinal cells under inflammatory conditions.

3.2 Effect of Robusta Green Coffee Extract on Pro-inflammatory TNF-α Production in CD11b+ Peritoneal Macrophages

Flow cytometry analysis revealed that the average percentage of TNF-α-producing CD11b+ peritoneal macrophages increased significantly following LPS induction. The normal control group showed a TNF-α expression of 26.44% ± 0.03, while the negative control group produced 63.82% ± 0.01. Treatment with RGC extract at concentrations of 30 µg/mL, 50 µg/mL, and 70 µg/mL resulted in TNF-α production levels of 44.61% ± 0.06, 49.87% ± 0.01, and 60.22% ± 0.03, respectively. These findings indicate that RGC extract, particularly at lower concentrations, reduced TNF-α production in peritoneal macrophages, with the most notable reduction observed at 30 µg/mL.

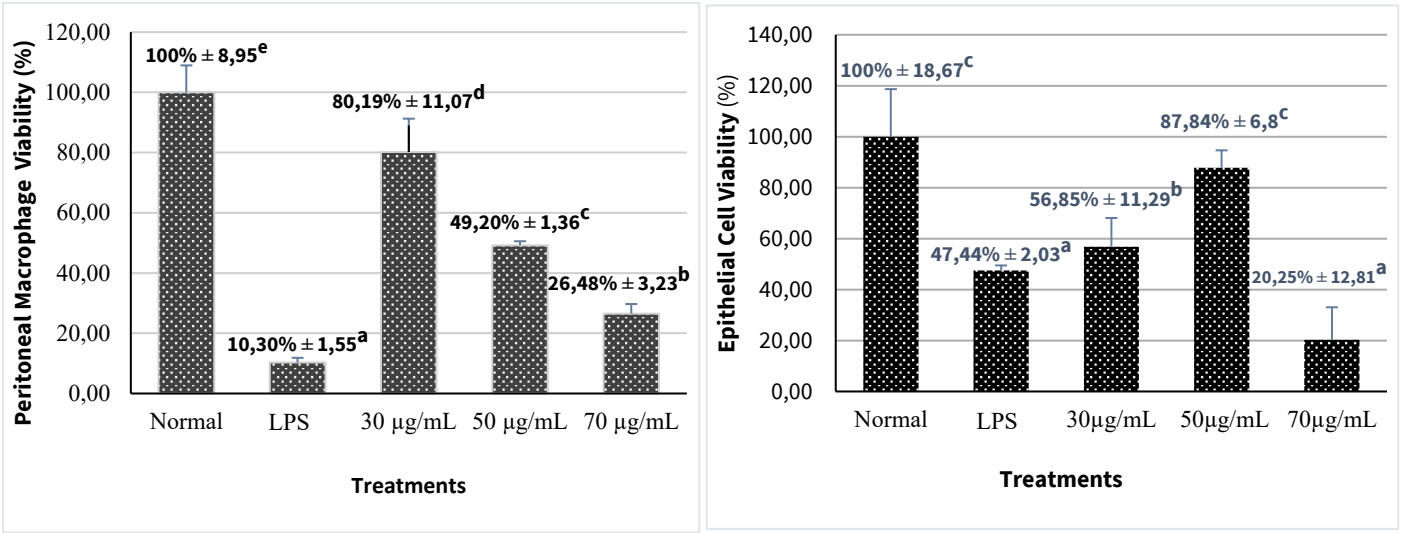

Figure 1. Comparisons of relative viability percentages of (a) peritoneal macrophages and (b) intestinal epithelial cells after LPS induction and treatment with Robusta green coffee (RGC) extract at various concentrations. Groups consisted of normal (N), negative control (C-, induced with LPS and treated with distilled water), and treatment groups receiving RGC extract at 30 µg/mL (P1), 50 µg/mL (P2), and 70 µg/mL (P3). Different letters indicate statistically significant differences between groups based on Duncan's Multiple Range Test (DMRT) at $p < 0.05$

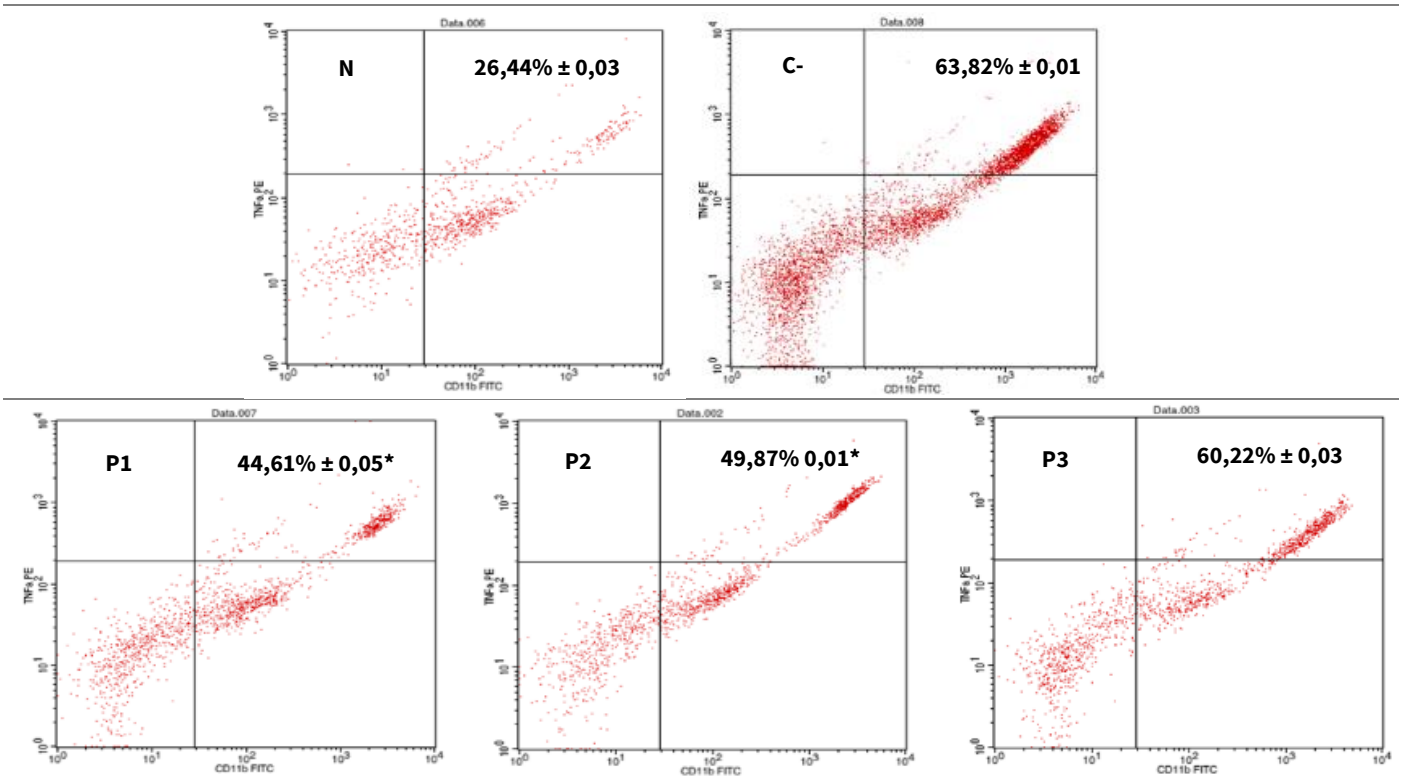

Figure 2. Flow cytometry analysis comparing the relative percentage of $CD11b^{+}TNF\text{-}\alpha^{+}$ macrophages under different treatment conditions. Groups included normal (N), negative control (C-, macrophages induced with 1 µg/mL LPS), and treatment groups receiving Robusta green coffee (RGC) extract at concentrations of 30 µg/mL (P1), 50 µg/mL (P2), and 70 µg/mL (P3). The normal group showed a TNF-α expression level of 26.44% ± 0.03, while the negative control group demonstrated a significant increase to 63.82% ± 0.01 following LPS induction. Treatment with RGC extract at 30 µg/mL, 50 µg/mL, and 70 µg/mL resulted in TNF-α expression levels of 44.61% ± 0.06, 49.87% ± 0.01, and 60.22% ± 0.03, respectively. Treatment with RGC extract at 30 µg/mL and 50 µg/mL showed a significantly lower TNF-α production compared to the negative control group ($p < 0.05$).

4. Discussion

In the context of Inflammatory Bowel Disease (IBD), such as ulcerative colitis and Crohn's disease, peritoneal macrophages and Intestinal epithelial cells play a central role as key immune sensors that recognize pathogens or pro-inflammatory stimuli such as lipopolysaccharide (LPS). Upon activation, these cells produce inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), which contribute to mucosal damage in the intestine. Intestinal epithelial cells, meanwhile, serve as the first line of defense against pathogenic microorganisms and maintain homeostasis through close interaction with the innate immune system (11). However, under IBD conditions, the integrity of the epithelial barrier is compromised, leading to increased intestinal permeability and excessive activation of macrophages. This interaction creates a self-perpetuating inflammatory loop through the secretion of pro-inflammatory cytokines, collectively exacerbating inflammation and worsening mucosal injury. To the best of our knowledge, no previous study has investigated the inflammatory interaction between primary peritoneal macrophages and intestinal epithelial cells in an in vitro LPS-induced model using in vitro.

The anti-inflammatory effects of RGC extract can be attributed to its high content of bioactive compounds, particularly chlorogenic acid (CGA) and caffeine (12). CGA, the major phenolic compound in green coffee, has been shown to reduce reactive oxygen species (ROS) production in LPS-stimulated macrophages and intestinal epithelial cells, thereby preventing oxidative stress and apoptosis (13). Additionally, CGA suppresses the NF-κB signaling pathway, leading to decreased expression of pro-inflammatory cytokines such as TNF-α. Caffeine also contributes by inhibiting NF-κB translocation, which downregulates key inflammatory genes like iNOS and COX-2, resulting in reduced production of TNF-α and prostaglandins (5, 14-15).

These findings are in line with previous studies reporting the anti-inflammatory activity of coffee extracts. Research on RAW 264.7 and THP-1 macrophages has demonstrated that coffee components can downregulate the expression and release of TNF-α and IL-6 in response to LPS (16-17). In this study, the RGC extract significantly reduced TNF-α production in LPS-induced macrophages, highlighting its potential as an effective natural immunomodulator and anti-inflammatory agent (18-20). The results of the present study further confirm the immunomodulatory potential of RGC extract by reducing TNF-α production in LPS-induced macrophages and enhancing the viability of both macrophages and intestinal epithelial cells under inflammatory conditions, supporting its potential as a functional anti-inflammatory agent.

5. Conclusions

Green Robusta coffee (GRC) extract effectively reduced TNF-α production in LPS-induced macrophages and enhanced the viability of intestinal epithelial cells. The optimal concentrations were 30 µg/mL for macrophages and 50 µg/mL for epithelial cells. However, the study is limited by its in vitro design, which may not fully capture the complexity of immune responses in living organisms. Therefore, comprehensive in vivo investigations and rigorously controlled clinical trials are urgently needed to validate these findings and determine the safety, efficacy, and pharmacodynamics of green coffee extract in the context of chronic inflammatory diseases.

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Supplementary Materials: The following supplementary materials are available: photographs of cell cultures, raw ELISA data, flow cytometry results, and statistical analysis outputs from SPSS. These materials provide additional supporting data related to the experimental procedures and findings presented in this study. [https://drive.google.com/drive/folders/1MvwL5MvtC_YE339PQR-pf_0sMnX-G4ty?usp=drive_link]

List of Abbreviations

IBD	Inflammatory Bowel Disease
NF-κB	Nuclear factor-κB
TNF	Tumor Necrosis Factor
TLR-4	Toll-like Receptor-4
LPS	Lipopolysaccharide
MyD88	Myeloid differentiation primary response 88
CD11b	Cluster of Differentiation 11b
iNOS	nitric oxide synthase
COX-2	cyclooxygenase-2
IL-6	Interleukin 6

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